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Prevalence of Papillomavirus, Epstein-Barr Virus, Cytomegalovirus, and Herpes Simplex Virus Type 2 in Urinary Bladder Cancer

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Recent epidemiological studies suggest that the risk for urological malignancies may be related to the exposure to infectious agents. Human Papillomaviruses type 16 and 18 (HPV 16, HPV 18), Epstein-Barr virus (EBV), cytomegalovirus (CMV) and herpes simplex virus type 2 (HSV-2) have been suggested previously as cofactors in the pathogenesis of some malignancies in humans. The present paper, the presence of HPV 16, HPV 18, EBV, CMV and HSV-2 genomes was investigated in a panel of 35 biopsies from urinary bladder carcinomas using the polymerase chain reaction (PCR). Sequences of EBV, HPV, CMV and HSV-2 genomes were detected in 34%, 31%, 11% and 9% of tissue samples respectively, while in 20% of patients we found more than one viral infection. Absence of viral genomes was found in normal bladder.To our knowledge, this is the first report concerning the association of EBV, CMV and HSV-2 with bladder cancer. This finding may raise the question whether such viral infection may contribute to development and progression of some types of urological malignancies in humans. ***J. Med. Virol. 55:262–267, 1998.***

KEY WORDS: HPV; EBV; CMV; HSV-2; bladder cancer; PCR

INTRODUCTION

Several human DNA viruses have been associated with tumor development. Compelling epidemiological and molecular data suggest that certain types of human papillomaviruses (HPV), as well as some members of the *herpesviridae* family, such as Epstein-Barr virus (EBV), human herpes simplex virus type 2 (HSV-2), and cytomegalovirus (CMV) may have an oncogenic role in cancer [Koffa et al, 1995].

HPV comprise a large group of small viruses with type-specific tropism for cutaneous or mucosal epithelia. These viruses on the basis of epidemiological or molecular studies have been implicated in several human cancers, particularly in tumors of the cervix, the anogenital region, the skin, the head and neck [zur Hausen, 1994]. In recent years a strong association has been demonstrated between infection with certain types of HPV, notably types 16 and 18 and the development of invasive cervical carcinoma [zur Hausen and de Villiers, 1994]. More recently, HPV types 16 and/or 18 genomic sequences have been identified in the urinary tract of female patients with recurrent and persistent urethritis and cystitis [Aglianò et al., 1994a] and in transitional tumors of the urinary bladder [Aglianò et al., 1994b].

EBV is a human herpesvirus which has been linked to Burkitt's lymphoma and nasopharyngeal carcinoma [Henle and Henle, 1985; Miller, 1990; Niedobitek et al., 1994; Chao et al., 1996]. In addition, EBV has been also associated with Hodgkin's disease [Weiss et al., 1989; Tomita et al., 1996], some tonsil [Brickacek et al., 1984], tongue [Raab-Traub et al., 1987], laryngeal [Kiaris et al., 1995], parotid [Saemundsen et al., 1982; Nagao et al., 1996], gastric [Shibata and Weiss, 1992; Hatsunou et al., 1996], and pulmonary neoplasias [Higashiyama et al., 1995], however the evidence is not conclusive. EBV has also been found to replicate in cervical epithelium and genomic sequences have been detected in cervical biopsies from patients with invasive carcinoma, thus suggesting a possible role in genital cancer [Landers et al., 1993; Wong et al., 1993]. EBV DNA has been also detected in the urethra of men with gonorrhoea, raising questions concerning the spread of this virus to the urogenital tract [Israele et al., 1991].

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Other sexually transmitted herpesviruses, including HSV-2 and CMV have been suspected of causing cancer. Epidemiologic data suggest that HSV-2 may interact with HPV in the pathogenesis of some tumors of the uterine cervix [zur Hausen, 1982].

CMV, like other DNA viruses, has been shown to transform morphologically different cell lines in vitro [Jariwalla et al., 1989; Razzaque et al., 1991]; and the immediate early (IE) gene products of CMV has been documented to transactivate cellular oncogenes *fos, jun* and *myb,* suggesting a possible oncogenic potential in vivo [Boldogh et al., 1991]. Indeed the role of CMV in human cancer has been postulated, even if the existence of a strict correlation between CMV infection and tumor development has not been well documented [Shen et al., 1993;Shen et al, 1994; Koffa et al., 1995].

Recent epidemiological features suggest that the risk of some urological malignancies, and genital neoplasias may be related to the exposure to infectious agents. Studies on the pathogenesis of bladder cancer seem to support this hypothesis, especially in those patients whose tumors show a clear tendency to multicentricity, papillary mode of growth and high recurrence rate [Kroft et al., 1994].

The detection of HPV in urethral condylomata, urine and seminal fluid first suggested the possibility that HPV infection may represent a risk factor for urogenital cancer development, then the finding of HPV genome or proteins in a variety of bladder tumors led to the consideration of a carcinogenic role for HPV also in the bladder [Bryant et al., 1987; Bryant et al., 1991; Aglianò et al., 1994b]. Thus, the pathogenesis of urogenital cancer may be postulated to follow two different pathways, being the first involving DNA tumor virus integration, and being the second independent of viral infection [Reznikoff et al., 1993].

Despite the increased number of reports concerning HPV identification in bladder cancer specimens, the presence and eventually the role of other known oncogenic viruses has been never investigated in urological malignancies. To obtain further information, the presence of HPV16, HPV18, EBV, HSV-2 and CMV genomic sequences was sought in 35 biopsies from bladder tumors of different staging and grading by the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Sample Collection and DNA Extraction

A total of 35 biopsy tissues of urinary bladder transitional carcinomas were obtained by surgical transurethral resection (TUR-B, 30 cases) or radical cystectomy (5 cases) of 29 male and 6 female patients (age 27–81; mean age = 67) attending the Cristo Re Hospital of Rome. Tissues were frozen in liquid nitrogen and stored at –80°C until use. All the tumors were diagnosed histologically as transitional cell carcinomas (TCC) ranging from G1 to G3 with respect to tumor grading, and classified clinically as Ta to T3.

DNA was isolated according to standard methods including proteinase K treatment in the presence of 1% SDS, phenol-chloroform extraction and ethanol precipitation.

PCR Amplification

For each sample five reactions were carried out in order to detect the presence of HPV16, HPV18, EBV, CMV and HSV-2 genomes. PCR was undertaken in a final volume of 100 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl, 200 mM of each dNTP, 2.5 units of *Taq* polymerase (Polymed), 1 μg of DNA sample and 50 pmol of each specific primer.

Sequences of primers and probes and the amplification conditions for HPV16, HPV18, CMV and EBV have been described previously [Gradilone et al., 1996]. PCO3 and GH21 [Saiki et al., 1988] were the primers utilized for β-globin gene amplification; the size of the fragment amplified by this pair of primers was 250 bp.

Positive controls in the PCR experiments included DNAs from CaSki cell line for HPV16, HeLa cell line for HPV18, AD169 infected fibroblasts for CMV, Raji cell line for EBV and HSV-2 infected HEp-2 cells for herpes simplex virus type 2. In each PCR experiment we included a sample without DNA as a negative control.

The expected size of the amplified sequences in the HPV16, HPV18, CMV, EBV and HSV-2 genome were 576, 360, 239, 337 and 391 bp respectively. Sequences of primers and probes for HSV-2 were: sense primer: 5′ AGGCCTACCAACAGGGCGTG 3′, nucleotides no. 966–985; antisense primer: 5′ CCTGGATCGACGGGATGTGC 3′, nucleotides no.1231–1250; probe: 5′ AACGCCACGCAACCCGAACTCGTTCCGGAAGA 3′, nucleotides no. 1133-1164.

The PCR amplification conditions used for HSV-2 were: denaturation step at 94°C for 1 min., annealing step at 60°C for 1.5 min. and primer extension step at 72°C for 3 min. The final extension step was prolonged for another 7 min. The PCR was run for 40 cycles of amplification in a Perkin Elmer-Cetus Thermal Cycler 480.

All the recommended precautions were taken in order to avoid contamination and the preparation of the reaction mixture and analysis of amplified products were carried out in separate rooms.

Analysis of the Amplification Products

Twenty microliters of the amplification products were electrophoresed on 2% agarose gel and the DNAs were then transferred to Hybond N membrane (Amersham) and fixed by UV irradiation for 5 min. Filters were hybridized with 10^6 cpm/ml of the specific 5′^{32}P-end-labeled oligonucleotide probe in a solution of 6× SSC, 10× Denhardt's solution and 0.5% sodium dodecyl sulfate (SDS). Filters were then washed in 1× SSC, 0.5% SDS at 60°C and autoradiographed on XAR films (Kodak) at –70°C.

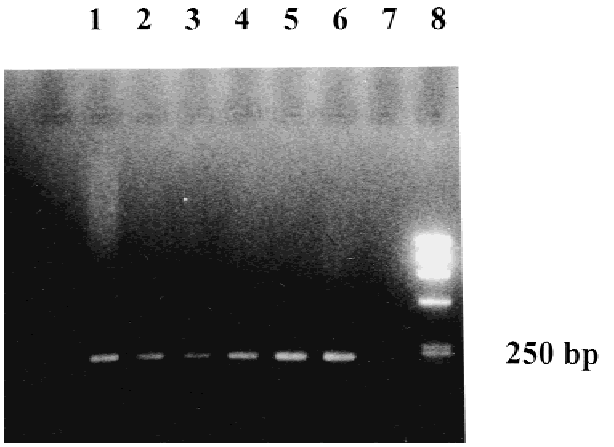

Fig. 1. Electrophoretic analysis of PCR products obtained with CO3 and GH21 primers. Lanes 1–6: urinary bladder cancer samples, negative for the presence of HPV, EBV, CMV, HSV-2 genomic sequences by PCR, but suitable for β-globin amplification. Lane 7: negative control (PCR reaction without DNA); Lane 8: DNA standard size marker (PhiX 174 DNA *Hae*-III digested).

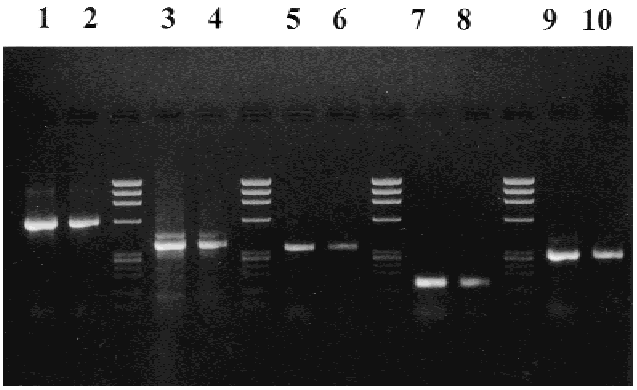

Fig. 2. Electrophoretic analysis of HPV 16, HPV 18, EBV, CMV and HSV-2 amplification products. Lanes 1, 3, 5, 7, 9: positive controls for HPV 16, HPV 18, EBV, CMV and HSV-2 respectively; lanes 2, 4, 6, 8: bladder cancer specimens positive for HPV 16, HPV 18, EBV, CMV and HSV-2 respectively.

RESULTS

Prior to investigation for the presence of HPV16, HPV18, EBV, CMV and HSV-2 genomic sequences by PCR, all specimens from urinary bladder cancer were examined with β-globin primers to assess DNA quality. All samples were found suitable (Fig. 1). After amplification by PCR and Southern blot hybridization of the amplified products, 21 of 35 cancer biopsies (60%) were found positive for the presence of viral genome, while viral DNA was not identified in the remaining 14 samples. The PCR conditions, which were different for each virus, had similar sensitivity for each system used, so that each virus was detected with the same sensitivity.

Sequences of HPV16 genome were detected in 6/35 patients (17%), all males; HPV18 was found in 5/35 patients (14%), 2 males and 3 females; EBV sequences were detected in 12/35 patients (34%) 9 males and 3 females; CMV was found in 4/35 patients (11%), 2 males and 2 females; HSV-2 sequences were detected in only 3/35 patients (9%), 2 males and 1 female (Fig. 2 and 3).

In 7 patients (20%) more than one infection was found; in 2 patients EBV/HPV18, in 1 patient CMV/HSV-2/HPV16, in 1 patient EBV/HPV16, in 1 patient CMV/HPV18 and in 2 patients HSV-2/EBV.

No significant difference was found overall for viral infection in tumors of different staging and grading. Interestingly, a significant difference was found for viral infection among males and females, being 5 of 6 females examined (83%) positive for one or more viral types, while positive males were 15/35 (42%). Figure 4 shows the incidence of viral infections in these patients.

Furthermore, 10 autopsy non-neoplastic urinary bladder tissue samples from subjects who had died without any bladder disease were tested for the presence of HPV, EBV, CMV, and HSV-2 infections; all the controls gave negative results (data not shown).

DISCUSSION

HPV and EBV are the human DNA viruses detected most often in a range of specific tumors (HPV in cervix carcinoma and EBV in lymphomas and nasopharingeal carcinomas), whilst CMV and HSV-2 have been demonstrated to display an oncogenic potential in vitro and have been proposed as cofactors for the development of cervical cancer [Kjeer, 1993; de Sanjose', 1994; Koffa, 1995].

While HPV infection has been detected previously by several investigators in urinary bladder cancer, there are no reports concerning the prevalence of EBV, CMV and HSV-2 genomes in bladder neoplasias.

The experiments described above were designed to investigate the prevalence of different DNA viruses (HPV, EBV, CMV, HSV-2) in neoplastic tissues from the urinary bladder. Among the numerous methods for detecting DNA sequences in tissue biopsies, the in vitro gene amplification technique of PCR was chosen followed by electrophoresis and hybridization with internal oligonucleotide probes, because it represents the most sensitive and specific method. Numerous negative controls were included in each experiment to rule out the possibility of contamination or carry over. All the negative controls were negative, thus excluding the possibility of contamination.

Twenty-one out of thirty-five (60%) cancer biopsies were found positive for viral infections, and in particular 31% positive for HPV and 34% positive for EBV. These findings raise the question whether these viruses may contribute to malignancy; indeed it remains very difficult to establish a causal relationship between infection with a specific virus and the development of a tumor.

Nevertheless, a working model of human bladder

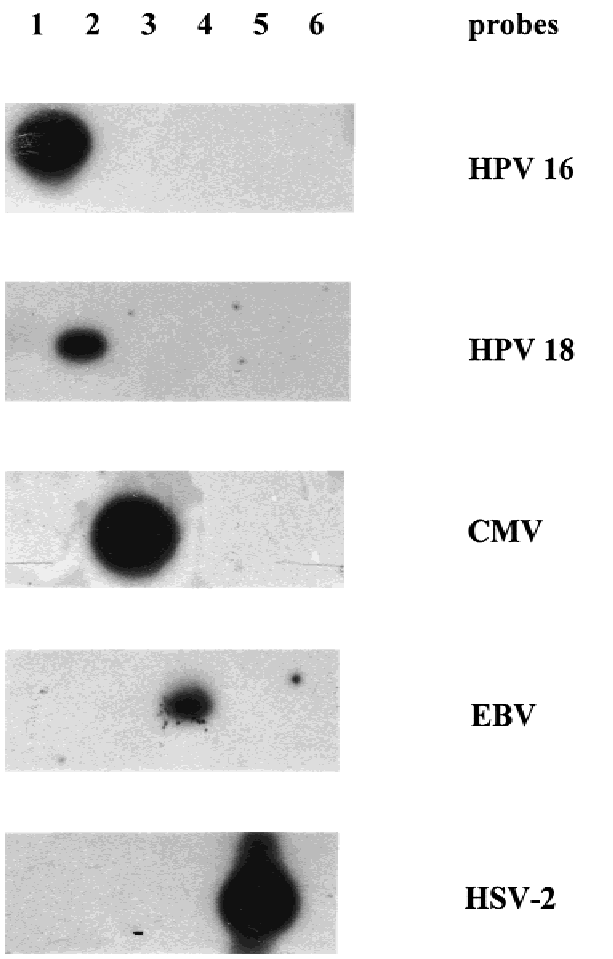

Fig. 3. Autoradiography of PCR products blotted and hybridized with ^{32}P-oligonucleotide probes specific for HPV 16, HPV 18, CMV, EBV, and HSV-2. In each filter: lane 1, sample positive for HPV 16; lane 2, sample positive for HPV 18; lane 3, sample positive for CMV; lane 4, sample positive for EBV; lane 5, sample positive for HSV-2; lane 6, sample negative for viral infection.

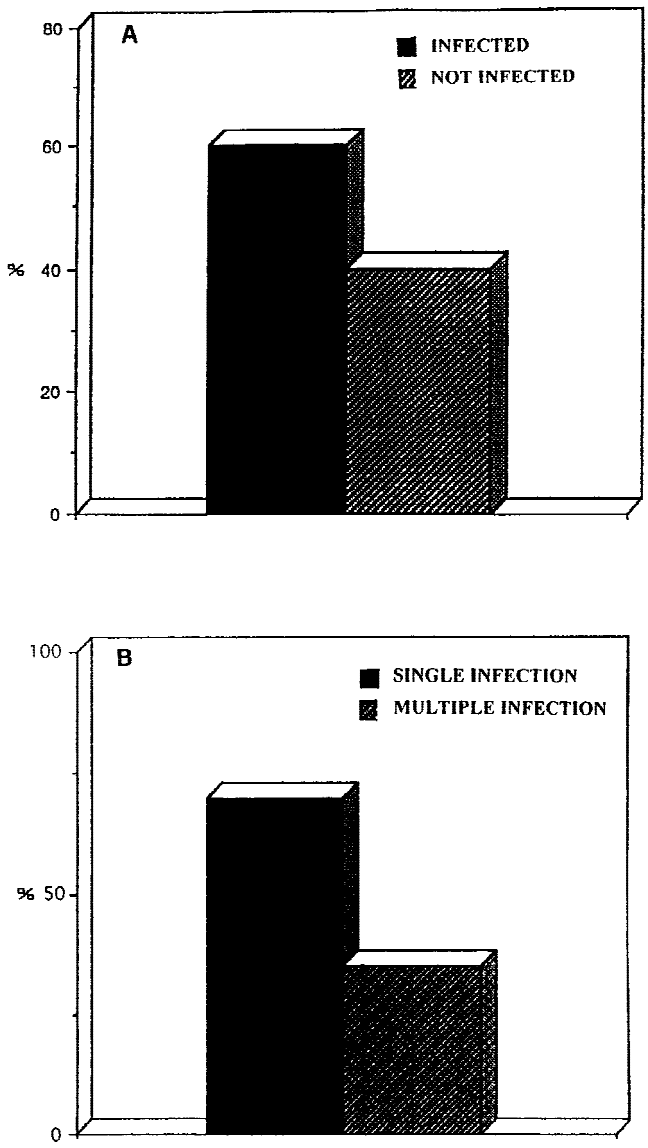

Fig. 4. Percentage of viral infections in urinary bladder cancer (Panel A). Percentage of multiple infections in urinary bladder cancer (Panel B).

carcinogenesis has been proposed by Reznikoff [1993], that takes in to account the possibility of existence of at least two pathways to uroepithelial tumorigenesis in vivo. The first pathway involves mutational activation of cellular oncogenes and inactivation of tumor suppressor genes. The second one, which involves HPV genome integration and E6 and E7 viral oncoproteins expression, concerns the lack of both p53 and pRB regulatory functions, leading to an extended lifespan of the infected cells. These cells would be at high risk for accumulation of the additional genetic changes required for immortalization and malignancy, since they are not able anymore to undergo apoptosis under genotoxic stimuli. Thus, chemical carcinogens or other DNA damaging agents would accelerate tumor progression. This hypothesis finds support in the long latency between HPV infection and development of cancer.

Also EBV is characterized by latency between infection and neoplastic transformation, and it is possible to postulate that EBV infection may interfere with apoptosis under genotoxic stress. The gene products of EBV, in contrast to HPV oncoproteins, do not seem to affect p53 and pRB functions, although are capable to inhibit apoptosis and extend the lifespan. Indeed, the BHRF1 protein (*Bam* HI-H rightword reading frame 1) presents about 38% homology with Bcl-2, the product of a cellular gene involved in the regulation of programmed cell death; *bcl-2* cDNA, when transfected in human B cells, protects them from apoptosis. Moreover the EBV latent membrane protein 1 (LMP-1) has been shown to up-regulate the cellular protein Bcl-2, leading to enhanced cell survival. Actually, *bcl-2* gene product is supposed to contribute to oncogenesis giving the cells that overexpress the protein a survival advantage over normal cells.

A previous study on the carcinogenesis of urinary bladder [Gazzaniga et al., 1996] showed the expression

of *bcl-2* in superficial uroepithelial tumor and its absence in adjacent normal tissue suggesting its possible implication in the initiation of the multistep process of bladder cancer development. It is postulated therefore that if EBV is involved in urinary bladder carcinogenesis its role may involve the deregulation of cellular apoptotic pathways. To confirm this, further studies on the expression of either BHR-F1, LMP1 EBV genes as well as *bcl-2* gene family are required.

The finding that 14% of the samples examined contained both EBV and HPV, or EBV and HSV-2 sequences raises the possibility of synergism between these viruses in bladder carcinogenesis. Such synergism has been already suggested in other human tumors, as some epithelial malignancies of the anogenital tract. In particular, CMV and HSV-2 involvment in the etiology of human cancer has been already demonstrated mainly in cervical carcinoma, where their presence has been found together with high risk HPV sequences by different groups. Their presence, in 11% and 9% respectively of the bladder tumors examined may suggest some role in the development of some urological malignancies, but further investigation with a larger number of patients are required.

In conclusion, the data show the presence of DNA tumor virus genomes, mainly HPV and EBV, in tumors of the urinary bladder, independently of histologic grading, clinical staging and gender of the patients. None of the DNAs extracted from normal bladders showed the presence of viral genomes by amplification in the same conditions. Further investigations are required in order to establish whether the finding of such viral infections in bladder tumors just represents an occasional event, or alternatively may play some role in the development and progression of some urological malignancies in humans.

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